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14. ABSTRACT

We implanted human prostate carcinomas either shortly after collagenase digestion or as tissue fragments obtained at surgery. We also genetically characterized tumors at harvesting and after growth in the murine host to determine if the proposed model maintains its initial genetic characteristics. After several trials in the orthotopic and subrenal capsule loci, we have implanted and begun to characterized by TMPRSS-ERG FISH and aCGH, 22 human primary prostate tumors. Results thus far show that the tumor take of prostate cancer in the subrenal capsule of mice is about 60%. Almost 80% of high grade human prostate tumors grow, whereas tumors with intermediate grade of differentiation are favored to grow if they show a high proliferation rate. By measuring serum PSA levels we can discriminate mice in which tumor xenografts grow from those in which growth of xenografts failed. Preliminary results showed that subrenal capsule xenografts maintain the same genetic background of the original parental human tumors from which they are derived. We are currently in the process of completing the genetic analysis of human tumors (with relative normals) and xenografts, to finalize our results.

15. SUBJECT TERMS
Organ-Confined Prostate Cancer, Xenographs, Orthotopic, Gene Expression Profiling, Single Nucleotide Polymorphism

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Introduction

Currently, primary human tumor material is insufficient due to small size, multifocality and difficulty of visualization at macroscopic examination. This essentially prevents extensive studies aimed at distinguishing indolent and aggressive organ-confined prostate cancers. Understanding the molecular alterations governing tumorigenesis and cancer progression is the first step necessary for the design of effective and targeted therapies. For this reason, in recent years considerable efforts have been devoted to generate clinically relevant models of prostate tumors. As a result, a number of cell lines and in vivo models have been developed. To date, however, there is no model in which all aspects of human PCa progression can be mimicked.

It is important to note that the overwhelming majority of organ-confined human prostate cancers display a luminal phenotype, characterized by expression of androgen receptor (AR), PSA and luminal-type keratins. The established human cell lines most commonly utilized in prostate cancer research (LNCaP, DU145, PC-3, LAPC-4 and LAPC-9) are derived from metastases and only few of them are androgen-sensitive [1, 2]. In the last 20 years a variety of methodologies have been attempted for the purpose of establishing primary epithelial cell cultures displaying a luminal phenotype reflective of the human primary prostate tumor of origin, but attempts have been largely unsuccessful. The possibility exists that small populations of contaminating non-malignant cells (invariably present in the human tissue sample) can populate and ultimately replace transformed cells in vitro. When transformed cells are indeed obtained from prostate primary adenocarcinomas they rarely form tumors in nude mice. Finally, primary cells from prostate cancers are mostly diploid and do not show relevant chromosomal alterations [3].

In recent years many mouse models of prostate cancer have been generated by injecting established cell lines (mostly LNCaP and PC-3 sublines) subcutaneously or orthotopically [4-7], while a few mouse xenografts have been established with human benign and malignant prostate tissues in both orthotopic and subrenal capsule sites [8, 9]. Experimental evidence suggests that orthotopically transplanted tumors may be the most appropriate system because of the potential to reproduce the microenvironment and metastatic patterns of human prostate cancer [10]. In addition, the relationships between nerves, stroma and epithelium in prostate cancer seem to be relevant as interactive processes requiring signaling systems and regulatory mechanisms that play major roles in the development, the local progression and subsequently the metastatic diffusion of prostate tumors [11-13]

We proposed to graft directly primary localized human prostate tumors samples orthotopically in immunodeficient mice and also to use these tumors for purifying to homogeneity in vitro epithelial cells showing a malignant behavior as assessed by growth in soft agar. This system, capable of mimicking the morphology, microenvironment, growth patterns and dissemination of human tumors will provide a new preclinical model to optimize therapeutic protocols and drug validation.

Body

Specific Aim 1. To collect fresh human prostate tissue samples and isolate primary prostate epithelial cell cultures for generating xenografts in nude mice (months 1-18)**.**

To accomplish this task, in the first year of the grant we had set the best technical conditions to manipulate and implant either fresh human prostate tissue or cells in immunodeficient mice (nude) in the orthotopic site.

Eight primary epithelial cell cultures and cell suspensions derived from 5 human primary tumors by collagenase digestion were mixed with matrigel and injected orthotopically in 34 mice. Three months following surgery mice were sacrificed, and in some of the cases we found small foci of human epithelial cells dispersed in the mouse prostate stroma, often surrounding neural structures, but no tumor of macroscopically visible size grew. In other cases the injection caused an important inflammatory reaction localized at the prostate. Four prostate samples were instead mechanically

disrupted and implanted into the mouse prostate, but only normal human prostate tissue was recovered from the xenografts. This could suggest that either normal tissue was originally implanted (verisimilarly due to the common histological heterogeneity of prostate tissue and the small size of tissue fragments that could be implanted in the mouse prostate) or tumor epithelium failed to grow in the hostile mouse microenvironment.

In light of these results, we chose the subrenal capsule as an alternative site of implantation for human prostate tissues in mice. This site of implantation is well vascularized and, more importantly, is able to accommodate a bigger amount of grafted human tissue, compared to the prostate lobe. In fact, with this technique we were able to achieve a high tumor xenograft take.

<u>Among 22 human primary prostate tumors that were implanted in the subrenal capsule site of 55 immunodeficient mice, 15 (65%) tumors grew in the murine host, whereas only 3 (13%) underwent regression, such as calcification. In addition, in 4 cases (18%) we found that only benign tissue was represented in the xenograft.</u>

Beside the group of tumors, 10 paired normal tissue samples, dissected from the peripheral zone of the prostate, were originally implanted under the renal capsule of 20 mice, two per each tissue. Surprisingly, in 4 out of 10 normal tissues, after 3 months from implantation, we found florid glands mixed to stroma. Only stroma was found in the other 6 normal xenografts.

Histopathologic and immunohistochemical characterization of mouse subrenal capsule xenografts and original human tumors.

Mice were sacrificed after three months from implantation and, at the gross pathology, subrenal capsule implants of normal tissues showed rare surrounding blood vessels, whereas tumors were covered by a robust vascularization origined from the renal artery or other close big vessels, such as the intestinal arteries (Fig.1). The total mass of tumor tissue implanted in mice showed an increase of about 1.5 fold relative to the original size of the implanted tissue in all xenografts. However, two recent tumors not included in this study group, that were left to grow for 6 months in mice, showed macroscopically bigger xenografts with nodular surface (Fig.1). This result could suggest that the xenografts undergo a first phase of slow growth, followed by a more rapid increase in size, and it is consistent with what has been recently described by Marangoni et al [14] in breast tumors. We could hypothesize that the initial phase after implantation (at least one month in our experience) is mainly required for the tissue to get completely vascularized. During this time the benign component, and probably part of the tumor cells as well, can regress.

Remarkably, all tumor xenografts maintained the histopathologic features of the parental tumors. In fact, they not only showed the same histotype and grade of differentiation (assessed by Gleason score) (Fig.2), but also resulted in a mix of tumor, benign glands and stroma.
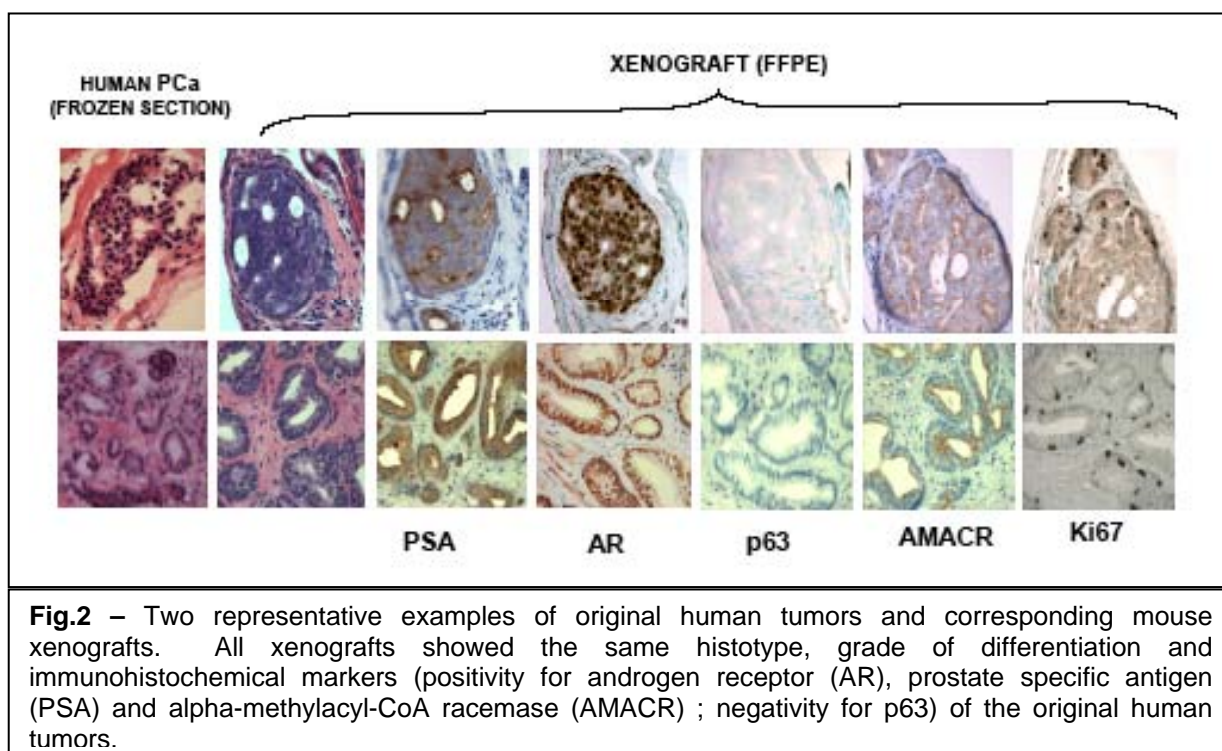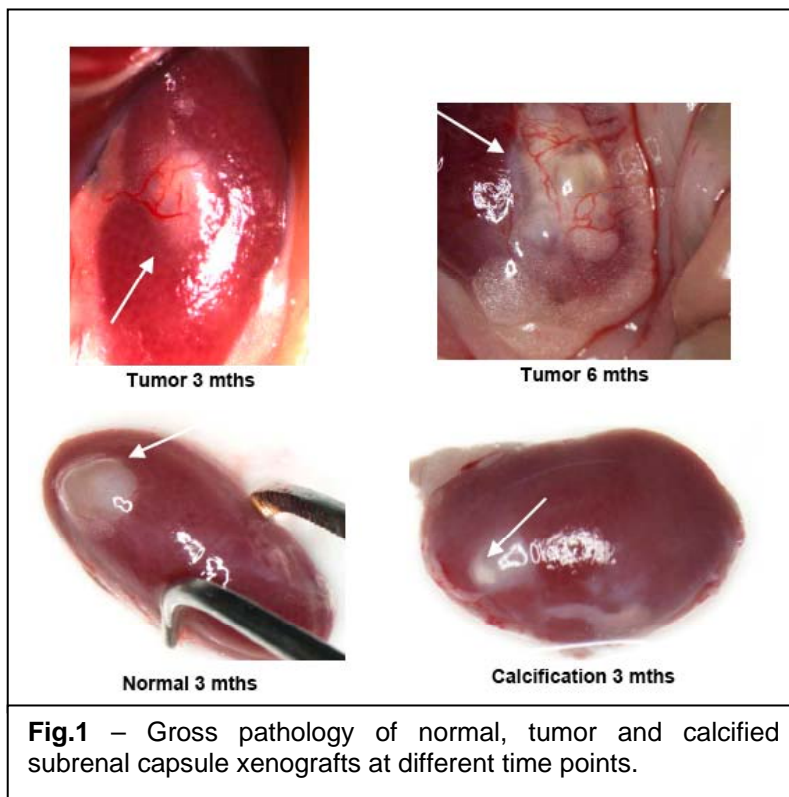
In order to investigate whether human tumors growing in immunodeficient mice were able to maintain the same phenotypic profile, paired original prostate cancers and xenografts were screened for canonical PCa markers by immunohistochemistry. Antibodies against androgen receptor (AR), prostate specific antigen (PSA) and alpha-methylacyl-CoA racemase (AMACR) [15] [16] were positive in all the xenografts, whereas p63 was negative (Fig.2), consistently with the original human tumors.

Human prostate tumors with different Gleason score (7- 9) and pathological stage (T2b-c, T3a-b) were originally implanted in the subrenal capsule site of both Nu/Nu (irradiated with 400 rads in unique dose 24 hours before surgery, or not irradiated) and SCID mice.

These two strains differ mainly because of their immunodeficient status: while athymic Nu/Nu mice lack T cells, NOD/SCID mice are lacking of both T and B cells. Irradiation of mice induces a severe depression of NK cell activity. Although it has been reported that more profoundly immunodeficient mice could enhance the tumor take [17], in our experience there was not significant difference in terms of xenograft growth rate between the two mouse strains. We believe, instead, that variables belonging to the human tumors themselves play a major role in determining the tumor take in mice. Importantly, in the group of human prostate tumors that were implanted, 80% of high Gleason (combined Gleason 8-9), but only 40% of Gleason 7 cancers, grew in mice. On the other hand, taking into consideration exclusively the pathological stage (although subgroups of tumors were too

small to draw definite results), a locally advanced condition such as pT3b stage wasn't sufficient to predict tumor xenograft's growth. In fact, only 50% of pT3 tumors successfully developed xenografts. Looking for other parameters of biological aggressiveness of our group of tumors, we compared Ki67 positivity percentage in each pair of human tumor and derived xenograft. Interestingly, although there was 100% concordance between them, when the parental tumor showed areas with high heterogeneity in proliferation, the xenograft maintained the highest proliferation rate, strongly suggesting that this could represent a major requirement for tumor growth in mice.

Fig.1 – Gross pathology of normal, tumor and calcified subrenal capsule xenografts at different time points.

Starting from this observation, we wanted to investigate whether the parental tumor proliferation affects xenograft take. To this purpose, we set an arbitrary Ki67 positivity cut-off of 5% and sub-grouped human PCa that grew in mice (n=14) and those of unsuccessful implantations (calcification or presence of stroma and some benign glands, n=7) with Ki67 higher or lower than 5%. We chose 5% as cut-off taking into consideration both the distribution of proliferation rate through our group of tumors and the fact that prostate cancer is among the most slow-growing tumors in humans. Of note, we found that tumor take in mice was associated to Ki67 expression >5% (Fisher's exact test, p=0.04).

Moreover, looking into the subgroups of tumors, while as expected high Gleason tumors were all highly proliferating, among Gleason 7 tumors only the ones with higher Ki67% grew.

Fig.2 – Two representative examples of original human tumors and corresponding mouse xenografts. All xenografts showed the same histotype, grade of differentiation and immunohistochemical markers (positivity for androgen receptor (AR), prostate specific antigen (PSA) and alpha-methylacyl-CoA racemase (AMACR) ; negativity for p63) of the original human tumors.

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Serum PSA levels in xenograft bearing-immunodeficient mice

In order to assess whether mice implanted in the subrenal capsule site bear successfully growing tumor xenografts without need to sacrifice them, we tested serum PSA levels in our mouse population. Importantly, mice don't produce PSA and therefore the antigen would be detectable in mouse serum

only if the grafted human tissue would be still able to produce and secrete it into the blood. Sera derived from mice implanted either with tumor or normal tissues were assayed for PSA, but only those cases for which at least two measurement were possible and gave consistent results were considered.

As expected, serum PSA values in mice implanted with normal tissue were low, in some cases undetectable (n=10, mean 1.02 ng/ml ±SD1), whereas in the tumor group PSA levels fell in a wide range (n=8, mean 13 ng/ml ±SD10.7). Importantly, there was a significant difference between the two groups (two sample T-Test, p=0.02) (Fig.3). This scenario nicely recalls the human

Fig.3 – Serum PSA levels were significantly different between tumor xenograft- and normal xenograft-bearing mice.

situation, where among healthy men serum PSA levels usually don't exceed the value of 4ng/ml, but in most cancers they increase with a large inter-patient variability.

Of note, PSA wasn't detectable in any mouse in which tumor propagation failed and tissue underwent calcification.

In order to investigate whether PSA levels could be informative of xenograft's growth, most of the mice were subject to bleeding monthly, and PSA measured. As a result, three month-PSA curves showed that serum PSA increased constantly every month when tumor xenografts were growing, while it was generally constant in case of normal tissue implantation and dropped to undetectable levels in case of calcification.

Specific aim 2. To perform gene expression profiling and SNP analysis on original prostate specimens and derived primary prostate cells before and after implantations in nude mice (months 6-34)**.**

In order to accomplish this aim and compare the genetic background of paired xenografts and original human tumors, we applied an array-based comparative genomic hybridization (aCGH). Tumor and normal epithelium were first independently isolated from formalin-fixed paraffin embedded (FFPE) tissues by Laser Capture Microdissection (LCM) and then processed for DNA purification and whole genomic amplification (WGA) (Fig.4). This methodology is challenging and often requires repeated reactions of DNA isolation and WGA from the same tissue sample prior to obtain a good quality DNA adequate for genome-wide analysis. In fact, FFPE DNA is usually highly degraded and the input material (tissue) from which DNA must be extracted is minimal (< 1 mm^2). Moreover, it seems that better results in genome-wide analyses of FFPE DNA can be achieved with aCGH compared to SNP arrays platforms. Because of these challenges, so far three paired xenografts and parental tumors with relative normal tissue have been processed for aCGH analysis (totally 12 DNA samples). Preliminary results from these samples support the hypothesis that human tumors maintain the main genetic alterations through their life span in mouse, without acquiring new large deletions or amplifications. As an example, the case called PCa 63 is shown in Fig.5. Genetic profiles of the parental tumor and the xenograft were superimposed and analyzed with CGH analytics software, revealing almost 100% concordance between the two samples.
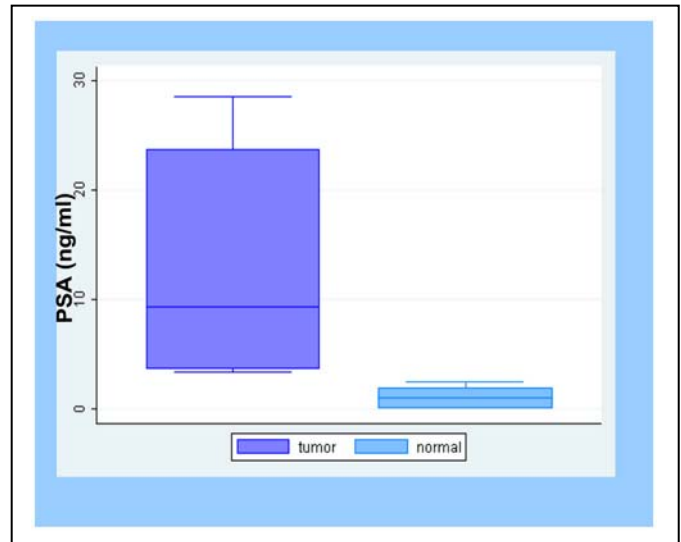
FFPE tissue **LCM** **Whole Genome Amplification**

Fig.4 - FFPE tissues were laser capture microdissected and then processed for DNA purification and whole genome amplification (WGA).

Fig.5 – Representative example of aCGH analysis on PCa63 and corresponding xenograft. Genetic profiles of the parental tumor and the xenograft were superimposed and analyzed with CGH analytics software, revealing almost 100% concordance between the two samples.
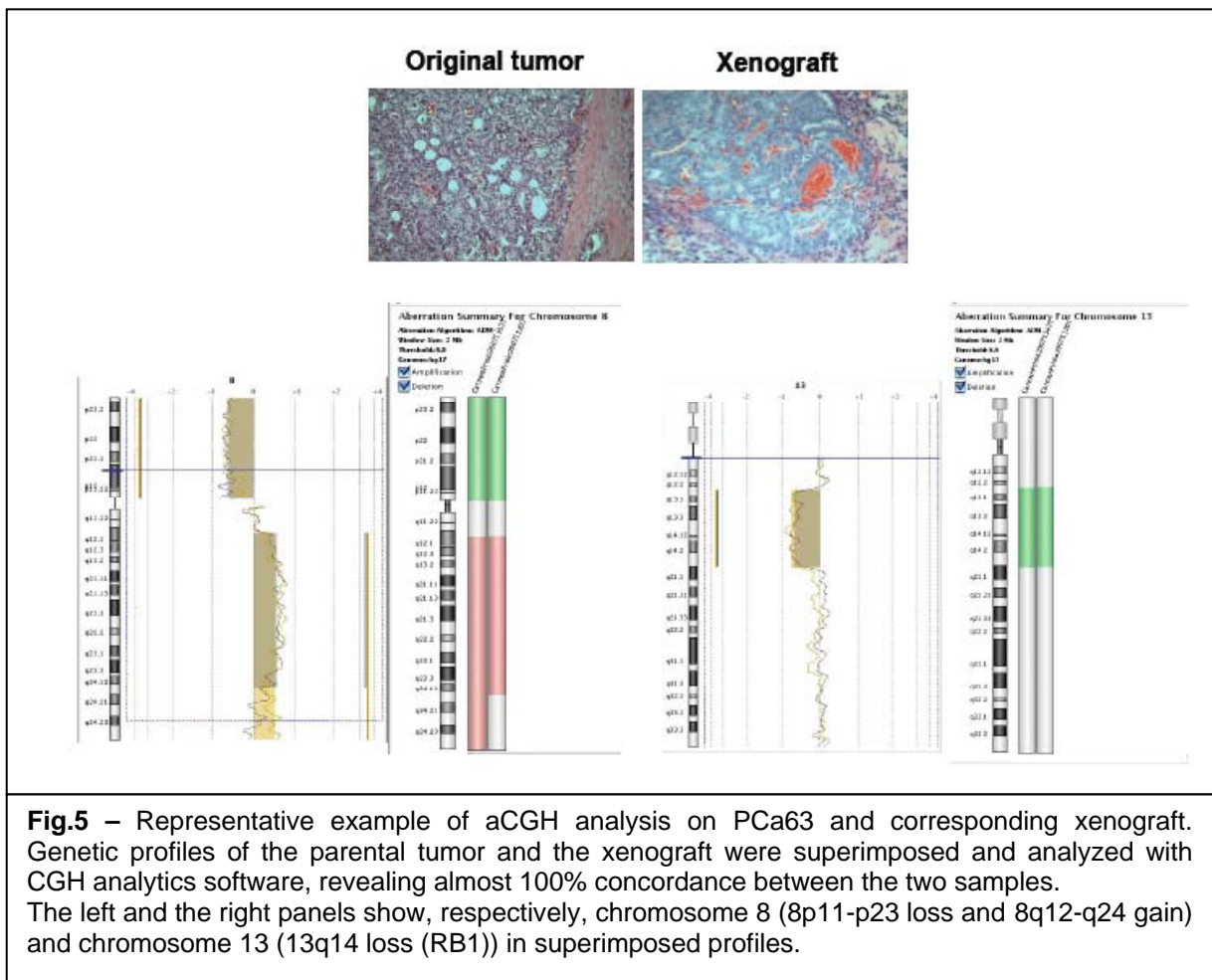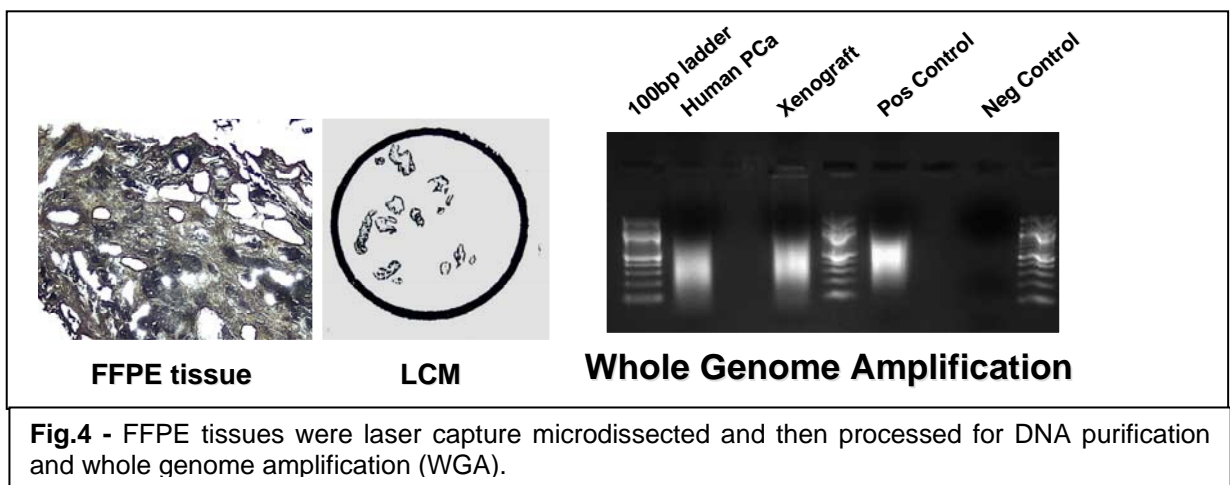The left and the right panels show, respectively, chromosome 8 (8p11-p23 loss and 8q12-q24 gain) and chromosome 13 (13q14 loss (RB1)) in superimposed profiles.

Specific Aim 3. To generate mouse orthotopic prostate cancer xenografts from engineered human primary prostate cell cultures (months 18-34)**.**

We will accomplish this task in the next year.

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Key research accomplishments

1) Subrenal capsule implantation of 22 human prostate tumor samples in 55 immunodeficient mice (irradiated and not irradiated Nu/Nu, NOD/SCID) with a high tumor xenograft take (>60%);

2) Identification of pathological and biological variables, such as grade of differentiation and proliferation rate, able to discriminate human prostate tumors that are more likely successful candidates to grow in immunodeficient mice;

3) Identification of serum PSA as a marker discriminating tumor xenograft- from normal xenograft-bearing mice

4) Validation of the subrenal capsule xenograft model as a system for *in vivo* propagation of human prostate tumor tissues that maintain a consistent genetic background through their life span in mice.

Reportable outcomes

XV SPORE meeting, Baltimore July 2007
Title: Establishment and Molecular Characterization of Mouse Xenografts of organ confined Human Prostate Tumors (**Oral presentation**)

Conclusions
Work in progress. The tumor take of prostate cancer in the subrenal capsule of mice is about 60%. Almost 80% of high grade human prostate tumors grow, whereas tumors with intermediate grade of differentiation are favored to grow if they show a high proliferation rate. By measuring serum PSA levels we can discriminate mice in which tumor xenografts grow from those in which growth of xenografts failed. Preliminary results showed that subrenal capsule xenografts maintain the same genetic background of the relative parental human tumors from which they are derived. We will complete the genetic analysis of a group of 10 pairs of human tumors (with relative normals) and xenografts, to finalize our results.
The development and characterization of such experimental model provide an invaluable resource to conduct new studies on the biology of primary localized prostate cancers and on the influence of the genetic background on drug resistance and sensitivity.

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